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METHOD IN CELL SCIENCE

Efficient method for generating nuclear fractions from marrow stromal cells

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Abstract Stem cells have received significant attention for their envisioned potential to treat currently unapproachable diseases. No less important is the utility of stem cells to serve as model systems of differentiation. Analyses at the transcriptome, miRNA and proteome levels have yielded valuable insights into events underlying stem cell differentiation. Proteomic analysis is often cumbersome, detecting changes in hundreds of proteins that require subsequent identification and validation. Targeted analysis of nuclear constituents would simplify proteomic studies, focusing efforts on transcription factor abundance and modification. To facilitate such studies, a simple and efficient methodology to isolate pure nuclear fractions from Marrow Stromal Cells (MSCs), a clinically relevant stem cell population, has been developed. The modified protocol greatly enhances cell disruption, yielding free nuclei without attached cell body remnants. Light and electron microscopic analysis of purified nuclei demonstrated that preparations contained predominantly intact nuclei with minimal cytoplasmic contamination. Western analysis revealed an approximately eightfold enrichment of the transcription factor CREB in the isolated nuclei over that in the starting homogenates. This simple method for isolation of highly purified nuclear fractions from stem cell populations will allow rigorous examination of nuclear proteins critical for differentiation.

Keywords Proteomic analysis · Nuclear fraction · Transcription factors · Differentiation

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Introduction

Significant research efforts are currently examining the potential of stem cells to treat disease. Disorders unapproachable or poorly managed with pharmaceuticals may ultimately yield to cell-based regenerative medicine. Clinical trials examining the utility of stem cells in the treatment of a range of disorders, from myocardial infarction to diabetes are currently underway. Marrow Stromal Cells (MSCs), a stem cell population of the bone marrow, figure prominently in a number of these trials. MSC-based therapies for neurological (Multiple Sclerosis) cardiac (heart failure) and skeletal (Osteogenesis Imperfecta) disorders are ongoing (http://clinicaltrials.gov).

Stem cells have also emerged as powerful tools for basic research. Toxicologists have employed Embryonic Stem Cells (ESCs) to investigate the genotoxic

effects of environmental pollutants (Chan 2008; Vinoth et al. 2008). In the pharmaceutical industry, stem cell-based assays been established as a low cost, high throughput approach to assess efficacy and toxicity in the early stages of drug development (Crook and Kobayashi 2008). Most importantly, stem cells have been exploited as in vitro models of differentiation. Varied methodologies have been employed to investigate the dynamics of stem cell differentiation at multiple levels (Ma et al. 2007).

Transcriptional analysis of stem cell differentiation using microarrays or sequential analysis of gene expression (SAGE) has proved fruitful, allowing simultaneous examination of thousands of known genes. (Ahn et al. 2004a, b; Bourne et al. 2004; Brandenberger et al. 2004; Calhoun et al. 2004; Hung et al. 2004; Qi et al. 2003; Sekiya et al. 2002, 2004; Stains and Civitelli 2003; Steidl et al. 2003). More recent studies have examined the role of untranslated miRNAs in stem cell differentiation (Mattick and Makunin 2005). Significant insights into mechanisms of differentiation have been elucidated through these transcriptional analyses.

Proteomic approaches to study adult stem cell differentiation have the potential to provide insights unattainable through contemporary transcriptional analysis. The ability to identify novel molecules and to analyze post-translational protein modifications are strengths of this approach. Despite these advantages, proteomic analyses of stem cell differentiation are comparatively rare. This reflects, in part, the complexity of the approach and the significant downstream labor required to identify regulated moieties. Recent efforts to streamline proteomic investigations have employed focused analysis of subcellular fractions (Dencher et al. 2007; Rabilloud et al. 1998; Sze et al. 2007; Wu et al. 2004). To be successful, such efforts must reliably segregate the fraction of interest from extraneous materials. In this report, we describe an efficient procedure for the isolation of nuclear fractions from MSCs, a prototypical stem cell population with clinical potential. Proteomic examination of purified nuclei may facilitate identification of relevant nuclear proteins involved in differentiation processes, unencumbered by superfluous cytoplasmic moieties. Studies examining the epigenetic changes that occur during stem cell differentiation may also benefit from this isolation procedure.

Materials and methods

Cell culture

Isolation of adult rat bone marrow MSCs and cell cultures were carried out using published procedure (Azizi et al. 1998). MSCs were obtained with a protocol and procedures approved by the Institutional Animal Care and Use Committee. Cells were cultured in 20% fetal bovine serum (FBS; Atlanta Biological; Norcross, GA) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Sciences; Carlsbad, CA), pH 8, at 37 °C and passaged at least ten times before being used for experimentation.

Preparation of nuclei from MSCs

All operations were performed at room temperature (RT) unless otherwise stated. Approximately 7.5×10^6 MSCs were harvested in PBS buffer and centrifuged at 700 rpm for 5 min (Hermle Z36c rotor). Resultant cell pellets were resuspended in five volumes of solution A (pH 7.3, consisting of 0.32 M sucrose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM $NaHCO_3$, 1 μg/mL leupeptin, 1 μg/mL Aprotin) with gentle trituration. Standard treatment samples were held on ice until needed. Modified protocol samples were placed in a −80 °C freezer for 30 min, followed by slow thawing at RT. Freeze-thaw cycle was performed two times. All samples (with and without freeze-thaw cycles) were lysed by trituration (12×) through a 22 G needle attached to a 3 cc syringe. Cell lysates were loaded on a sucrose gradient (10 mL each of 1.9 and 1.5 M of sucrose, 1 mM $NaHCO_3$) and centrifuged at 28,000 rpm for 1.5 h in a SW 28 rotor. Nuclear pellets were resuspended in 0.6 mL of solution B (pH 7.2, consisting of 0.32 M sucrose, 1 mM $NaHCO_3$), transferred to a 1.5 mL tube and centrifuged at 14,000 rpm for 25 min in JA-17 rotor. Pellets were again resuspended in solution A and stored at −80 °C.

Light microscopy of homogenized cell suspensions

Cell lysates generated following the standard protocol or the modified freeze-thaw procedure were examined by light microscopy prior to sucrose gradient

centrifugation to evaluate the extent of cell lysis, degree of subcellular disruption and integrity of the nuclei. Representative images were captured using a Zeiss Axiovert microscope and accompanying software. Analysis was performed independently three times with similar results obtained.

Light and electron microscopy of isolated nuclei

Nuclear pellets obtained following sucrose gradient centrifugation were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 2 mM Ca^{2+}, pH 7.3, for 45 min at room temperature. The pellets were rinsed thoroughly in the cacodylate buffer and then treated with 1% osmium tetroxide in the same buffer for 1 h. After further rinsing the pellets were stained *en bloc* with 1.5% aqueous uranyl acetate, dehydrated in graded ethanol and embedded in Spurr's epoxy resin. Thin sections were cut and stained with uranyl acetate and lead citrate.

SDS–PAGE and Western blot analysis

All procedures were performed at room temperature. Vertical slab SDS–PAGE was performed using a continuous 3.5–15% acrylamide gradient. Electrophoresis was carried out at 125 V for 20 h. Proteins were electrophoretically transferred to Immobilon-P membrane (Millipore) at 25 V for 20 h. The transfer buffer consisted of 25 mM Tris–HCl and 200 mM glycine (pH 8.3–8.6). The resultant membrane was blocked with 5% fat-free milk in 0.1% TBS-T (20 mM Tris–HCl, pH 7.4/0.1% Tween 20) for 1 h at room temperature, incubated with antibodies (0.5 μg/mL for anti-CREB) for another hour, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham) (1:5,000) for 45 min. Products were analyzed using the enhanced chemiluminescence Western blotting detection system supplied by Amersham.

Quantitation

The intensity of CREB bands on immunoblots was quantitated with a scanning densitometer (CliniScan, Helena Laboratories, Beaumont, TX). The CREB level in the total homogenate was assigned a value of 100%.

Miscellaneous

Protein concentration was determined by modified Bradford assay (Bio-Rad). All experiments were performed three times. Results were analyzed by student *t*-test. Representative results are shown in the figures.

Results

Effects of double freeze-thaw

First, we examined the effects of double freeze-thaw on cell disruption as a prelude to nuclear isolation. MSCs resuspended in lysis buffer were either kept on ice or subjected to two successive freeze-thaw cycles. All samples were then homogenized using a syringe affixed with a 22-gauge (22 G) needle. Initial experiments assessed cell disruption after 3, 6, 9, 12 and 15 successive triturations. Microscopic analysis demonstrated that 12 triturations resulted in maximal cell disruption without compromising the integrity of the nuclei (data not shown). All subsequent experiments routinely employed 12 triturations of the cell suspension.

Trituration of cell suspensions with and without freeze-thaw cycles resulted in cell lysis. However, cells subjected to the standard procedure were poorly disrupted, with the nucleus remaining physically attached to remnants of the cell body. In contrast, the addition of freeze-thaw cycles prior to trituration resulted in near complete disruption of the cell, with nuclei routinely separated from other cellular components (Fig. 1). Numerous nuclei could be observed and integrity did not appear to have been compromised. More complete cell disruption at this stage enhanced the subsequent yield of purified nuclei (see below).

Microscopic analysis

Nuclear preparations obtained following sucrose gradient centrifugation were colorless and near transparent, suggesting high purity devoid of contaminating cytoplasmic components. To determine purity and assess integrity of the nuclear pellets, samples were examined in detail via light microscopy (LM) and electron microscopy (EM). LM analysis indicated that the isolated preparations were homogeneous,

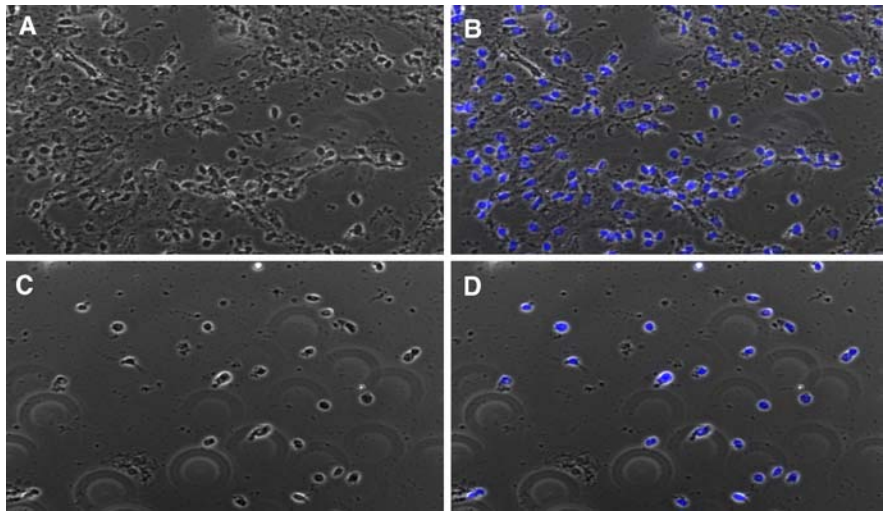

Fig. 1 Comparison of standard and modified protocols on cell disruption. MSCs lysed by trituration with or without freeze-thaw steps were examined microscopically. **a** Standard lysis protocol resulted in poor dissociation, with nuclei physically associated with remnants of the cell body. **b** Same field as in (**a**) with nuclei labeled with DAPI. **c** MSCs lysed using modified protocol contain well-isolated nuclei and enhanced fragmentation of non-nuclear components. **d** Same field as in (**c**) with nuclei labeled with DAPI. 20× magnification. *DAPI* 4′,6-diamidino-2-phenylindole

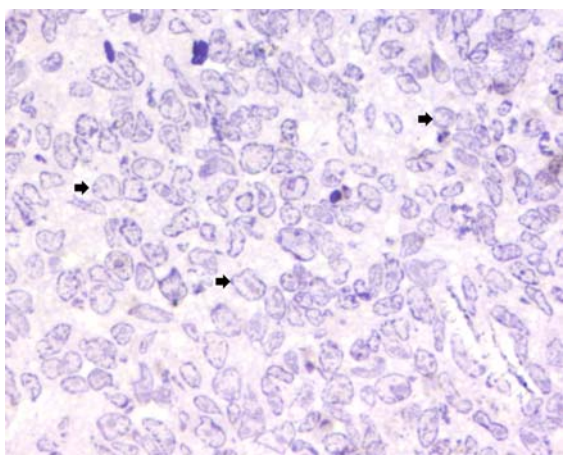

Fig. 2 Light microscopic examination of isolated nuclei. Light micrograph of isolated nuclei at 40-fold magnification shows a homogeneous preparation. *Arrows point* to three representative isolated nuclei with intact nuclear membranes

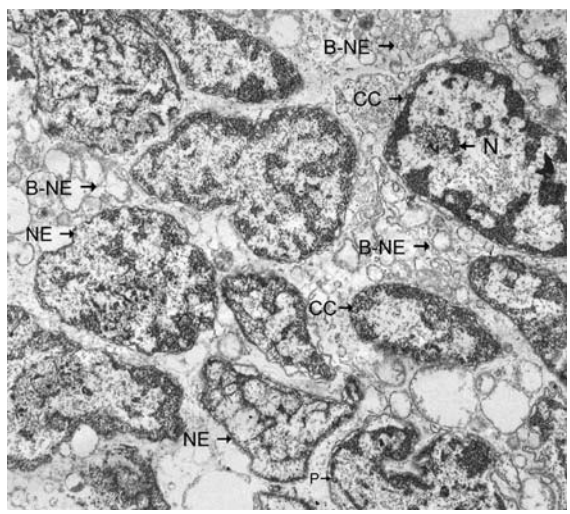

Fig. 3 Electron microscopic imaging of isolated nuclei. Electron micrograph of the isolated nuclei at 15,000-fold magnification shows intact nuclei with minimal contamination from cytoplasmic components. *CC* condensed chromatin; *N* nucleous; *NE* nuclear envelope; *P* nuclear pore; *B-NE* broken nuclear envelope

consisting almost entirely of nuclei without obvious contam-
ination. Minor contaminants consisted primarily of ruptured nuclear envelopes, with little evidence of cytoplasmic components (Fig. 2). At the EM level, the nuclear profiles appear intact and devoid of obvious preparative damage (Fig. 3). The nuclear envelope was well preserved, with no evidence of nucleoplasm loss through the nuclear pores. The isolated nuclei contained condensed chromatin, with defined nucleoli observed in some nuclear profiles.

Nuclear recovery

To determine the yield of the preparation, we determined the amount of protein in the isolated nuclei as compared to that in the homogenates. In three separate experiments, the yields were 1.90 mg nuclei/16.8 mg homogenates, 1.0 mg nuclei/7 mg homogenates and 2.5 mg nuclei/18.7 mg homogenates, respectively (Table 1). Nuclear preparations contained 12.97 ± 1.50% of the protein found in the initial lysates, suggesting 7.79 ± .94-fold enrichment of the nuclear proteins and concomitant removal of contaminating non-nuclear proteins.

Table 1 Yield of nuclear proteins isolated from adult bone marrow stromal cells

Experiment	Total proteins (mg)		% yield	Fold-enriched
	Homogenates	Nuclei		
I	16.8	1.9	11.3	8.84
II	7.0	1.0	14.2	7.04
III	18.7	2.5	13.4	7.48

Nuclei were isolated from MSCs in three separate experiments. The cells were subjected to double freeze-thaw treatment and were then homogenized for isolation of nuclei, as described in Methods. The mean ± SD of yield and fold enrichment from the three experiments were 12.97 ± 1.50% (p-value, < 0.0002) and 7.79 ± .94 (p-value $= 0.0002$), respectively

Nuclear protein enrichment

To begin analyzing the nuclear proteins in the isolated nuclei, we employed Western analysis to examine cyclic-AMP response element binding protein (CREB), a key transcription factor. Adult rat cerebral cortical nuclei served as a positive control. Anti-CREB antibody detected a 32 kDa protein in both MSC preparations and the cerebrocortical nuclear controls, consistent with the known molecular weight of CREB protein (Fig. 4). The antibody employed detected two isoforms of CREB in the cortical nuclei and one in the MSC nuclear fraction. The significance of this difference is presently unclear. Subsequent analysis revealed more than eightfold enrichment of CREB in the isolated nuclei over that in the starting total (Fig. 4).

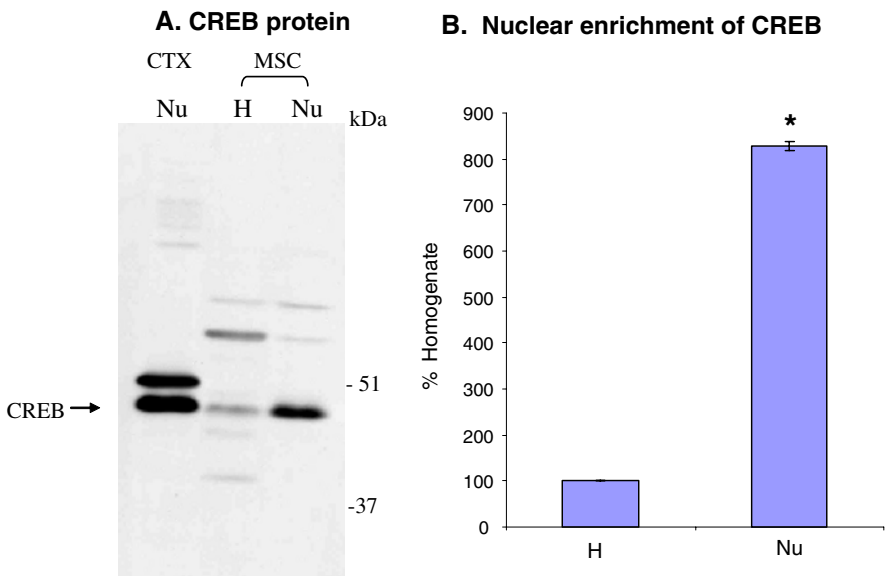

Fig. 4 Western blot analysis of CREB in homogenates and purified nuclear preparations. One hundred microgram each of MSC homogenates (H), isolated nuclei (Nu) and 30 μg of cerebrocortical (CTX) nuclei were analyzed by Western blot with anti-CREB antibodies (**a**). Western blot of CREB protein. *Arrow* indicates the position of CREB. Relative positions of molecular weight markers are shown. **b** Quantitation of CREB protein present in the homogenates and in isolated nuclei from three independent experiments, showing a concentration of greater than eightfold. The three enrichment values of 838, 819 and 830% were employed for calculation of the *error bar*

Discussion

Proteomic analysis has emerged as a powerful tool to study aspects of stem cell differentiation. Efforts in our laboratory investigating the differentiation of MSCs to putative neurons have revealed inherent challenges associated with proteomic approaches. In our studies, more than 500 unique protein spots are reproducibly detected at a single time point in the differentiation process. These novel entities likely represent products of genes expressed de novo as well as molecules subjected to post-translational modification during differentiation. Analysis of such a large number of molecules is daunting, restricting analysis to a subpopulation. With little or no data to indicate which molecules may be important in the differentiation process, this approach is very likely to exclude important proteins from further analysis.

To circumvent these difficulties some studies have eschewed whole cell proteomics, employing a more focused approach. Sze and colleagues performed proteomic analysis exclusively on proteins secreted by ESC-derived MSCs. This paracrine proteomic approach identified approximately 200 gene products, involved in such diverse biological events as vascularization and skeletal development (Sze et al. 2007). While protein identification and validation of expression still represents a significant challenge, this streamlined effort eliminated thousands of gene products from consideration.

We have undertaken an analogous approach to identify changes in nuclear proteins associated with stem cell differentiation. In support of this effort we have devised a novel approach to generate homogeneous nuclear preparations. Initially, we attempted to isolate nuclei from cultured adult bone marrow stem cells using an established protocol. Yield was routinely poor and preparations were not sufficiently pure to encourage further examination. Microscopic examination indicated that cell lysis was incomplete, suggesting that MSCs possess significant intrinsic mechanical strength that renders them resistant to disruption by traditional approaches. Indeed, it has recently been reported that MSC-like cells isolated from human umbilical cord blood are far less susceptible to osmotic lysis than all other resident cell types. This osmotic resistance appears to be due to cytoskeletal elements that enhance the mechanical stability of the MSC-like cells (Parekkadan et al. 2007). MSC-like cells can be isolated from multiple sources, including skeletal muscle, pancreas, adipose tissue, and placenta (Crisan et al. 2008). All may exhibit similar resistance to lysis.

To overcome this constraint, harvested MSCs in suspension were subjected to sequential freeze-thaw cycles prior to trituration. Using this approach, near complete disruption of freeze/thawed cells was achieved, while the nuclei remained intact. In parallel studies, we employed a dounce homogenizer rather than a needle and syringe to lyse the cell suspension. Again, freeze-thaw cycles clearly enhanced cell disruption, but final product was deemed inferior to that obtained through trituration (data not shown).

Following cell lysis, non-nuclear components of the lysate were removed and nuclei concentrated by centrifugation in a discontinuous density gradient (Maggio et al. 1963). Macroscopically, the nuclear fraction was homogeneous, appearing colorless and near transparent. Light microscopy of the preparations confirmed these observations, revealing highly concentrated and apparently intact nuclei with little evidence of non-nuclear contamination. Further evaluation via electron microscopy demonstrated that the vast majority of isolated nuclei were intact. Fragmented nuclear membrane was present in minimal quantities, indicating little damage occurred during isolation.

To further examine the utility of this approach, we compared the total protein content of whole cell lysates and purified nuclei. On average, nuclear isolates contained eightfold less protein than whole cell homogenates, suggesting significant reductions in contaminating cytoplasmic moieties and concomitant concentration of nuclear proteins. To confirm, we performed Western blot analysis on proteins derived from whole cell lysates and nuclear preparations. Quantitative analysis was performed to examine the relative concentration of CREB, a ubiquitously expressed transcription factor. Nuclear preparations contained 8.3-fold more CREB than whole cell lysates. This level of enrichment agrees nicely with the approximately eightfold decrease in total protein achieved by the subcellular fractionation procedure. Moreover, Western blot analysis suggests that the nuclear proteins isolated in this fashion are largely intact: the CREB-specific antibody recognized an entity of appropriate molecular weight without evidence of degraded products.

Analysis of the nuclear proteome seems particularly relevant to analysis of stem cell differentiation. Many studies have documented the dramatic changes in global gene expression that accompany stem cell differentiation. The expression levels of more than 100 proteins change greater than twofold during osteoblastic differentiation of MSCs (Zhang et al. 2006). Examination of such large numbers of potentially relevant genes and gene products can be daunting. Proteomic approaches are particularly susceptible to target overload, as significant downstream effort is required to identify protein species and confirm the observed changes in expression levels. However, proteomic approaches are unique in their ability to take advantage of post-translational localization of molecules, and focus subsequent analysis on discrete subcellular fractions. This approach has been employed with success to investigate the mitochondrial, golgi and secretory proteome (Dencher et al. 2007; Rabilloud et al. 1998; Sze et al. 2007; Wu et al. 2004). While microarray analysis has identified changes in transcription factors that correlate with a switch from pluripotency to differentiation, analysis of the nuclear proteome during stem cell differentiation has not been performed (Gunji et al. 2004). The simple methodology reported here will facilitate examination of the nuclear proteome during stem cell differentiation.

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